Self-assembled Multifunctional Nanoplexes for Gene Inhibitory Therapy

By: Fereshteh Azari, Marinella G. Sandros, Maryam Tabrizian

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Abstract:

Aim: To enhance the stability of siRNA while improving their therapeutic properties and visualization at the target site, a novel nanoplex system was developed. **Materials & Methods:** The designed nanoplex system involved functionalizing siRNA with near-infrared quantum dots and loading them into histidylated glycol chitosan (GC-His). **Results:** Colocalization studies revealed a twofold increase in siRNA uptake after encapsulation with GC-His and nanoparticles were localized in cytoplasm, suggesting that histidine promoted their dissociation from the endosomal membranes. Furthermore, as opposed to siRNAs treated with commercial transfection reagent, siRNAs loaded within GC-His showed a marked reduction (64%) of MDM2 protein expression 24 h after transfection. **Conclusion:** These findings concur that GC-His/siRNA-quantum dot nanoplexes are promising multifunctional vehicles for gene inhibitory therapy.

Keywords: glycol chitosan | imaging | N-acetyl-histidine | quantum dots | siRNA

Article:

RNAi is a post-transcriptional gene silencing process that is directed by short double-stranded RNA (siRNA, 21-23 nucleotides) [1-4] . siRNAs can assemble into complexes known as RNA-induced silencing complexes, which cleave complementary RNA molecules and inhibit expression of endogenous genes in a sequence-specific manner [5] . The use of siRNA approaches offer promising tools for cancer treatment. Inhibition of cancer growth has been achieved by targeting a variety of oncogenes using siRNA. The cellular oncoprotein, Murine double minute 2 (MDM2; also termed HDM2 in humans) plays a key role in tumor-radiosensitivity and is overexpressed in approximately 73% of breast carcinomas. MDM2 also associates with the tumor suppressor, p53 and downmodulates p53 function [6] . Inactivation of MDM2 using siRNA can not only activate p53 and suppress tumor growth, but it also enhances tumor sensitivity to radiotherapy [7] .

Unlike small molecule hydrophobic drugs, naked siRNAs are negatively charged macromolecules and cannot cross the cell membrane spontaneously and traffic into the cytoplasm of the target cells [8-10] . Delivery of such intact functional siRNAs into the cytoplasm remains a major drawback for RNAi-based therapy. As in DNA carriers, earlier therapeutics for enhancing cellular siRNA delivery employed either synthetic or viral vehicles; however, these approaches experienced various limitations and do not offer a good balance between gene silencing efficacy and toxicity [11-13] . One of the latest attempts to modify siRNA delivery involved hyaluronic acid (HA) electrostatically complexed with poly L-arginine, which, after mixing with siRNA spontaneously, self-assemble into polyplexes [14] . This HA-poly L-arginine delivery vehicle offered targeted delivery with low cytotoxicity. In another recent report, polyethyleneimine polymers have noncovalently been attached to the surface of mesoporous silica nanoparticles for enhanced delivery of siRNA [15] . Alternatively, Kim and coworkers have resorted to using nonpolymer-based vehicles, such as double-stranded RNA activated protein kinase R, without presenting any cytotoxicity [16] . While the above reports highlight significant advances in siRNA delivery, these formulations are not multifunctional in a true sense. In this article, the functionalization of *N* -acetyl-histidine on glycol chitosan (GC-His) will induce the proton sponge effect, leading to enhanced cytosolic release of siRNA, while the siRNA-conjugated quantum dots (QDs) provide means to monitor this process providing a highly multifunctional delivery system.

The advantage of chitosan is that it is a non-toxic, biodegradable cationic polymer with excellent bioadhesive properties [17,18] . Even though chitosan-based polyplexes offered excellent stability and protection for DNA in the cellular endosomal compartments, it failed to promote the release of DNA to the nucleus, which accounted for the low gene expression [19,20] . In order to overcome the latter limitation, herein, GC is used in the formulation of the nanoplex system, since it is soluble in water at all pH values, as opposed to chitosan that is only soluble in acidic aqueous solutions [21,22] and has been shown to form more stable monodisperse self-aggregated nanoparticles in water [23] .

While hydrophobically modified GC has been used for the delivery of DNA and drugs [23-27] , the major concern remaining is the fate of the nucleic acid after cellular internalization. Usually, internalized nanoparticles rapidly experience a drop in pH from 7.4 on the cell surface to 6.0 in the lumen of early endosomes and through their progression to late endosomes, the environmental pH is reduced to 5.0. As the imidazole ring of histidine is a weak base and under slightly acidic conditions (<6.0) can induce a proton sponge effect [25] , as a consequence, the endosome vesicles (acidic) swell due to an increase in their osmolarity and thus promote the release of siRNA into the cytosol. Histidylated nanoplexes like GC-His should offer many advantages, over previously reported delivery vehicles for siRNA such as very low cytotoxicity and the means to induce a proton sponge effect inside endosomal compartments and thus promote the release of their content into the cytosol, leading to greater gene knockdown.

In addition to introducing histidine in the design of the delivery vehicle, GC-His and streptavidin-coated near infrared (NIR) QDs functionalized with biotinylated siRNAs are included in the formulation to enhance the transfection and allocation of siRNA in the cytoplasm (Figure 1). Apart from streptavidin having minimal nonspecific binding to other proteins, the bond between streptavidin and biotin is very strong ($K_d = 10^{-14}$mol/l). The robustness of the binding ensures that siRNA-QD conjugates will remain stable under biological conditions, and its presence will be associated with the siRNA. Common tracking approaches often employ organic fluorophores for monitoring siRNA delivery. A few studies provide a platform that serves as a targeted and self-tracking vehicle for siRNA delivery using QDs. For instance, Tan *et al.* encapsulated siRNAs and QDs in chitosan nanoparticles (60 nm) and showed that the release of siRNA into the cytoplasm only started to take place after 2 days [28] . In a more recent study, Klein *et al.* used silicon QDs covalently decorated with 2-vinylpyridine to facilitate the electrostatic assembly of siRNAs on the surface [29] . These proposed delivery setups did not allow clear-cut detection of siRNA inside the cell after it is disassembled from the delivery platform and only employed QDs emitting in the visible region. As an alternative to these shortcomings, NIR emitting QDs is utilized in the formulation of the proposed nanoplex system to facilitate visualization of gene allocation and overcome cell and tissue autofluorescence [30] .

FIGURE 1 IS OMITTED FROM THIS FORMATTED DOCUMENT

Figure 1. Synthesis of nanoplex *N* -acetyl-histidine on GC-His with a siRNA-quantum dot conjugate.

Methods

Glycol chitosan (MW 200 kDa, degree of acetylation 9-12%), *N* -acetyl histidine (NAcHis), *N* -(3-dimethyl aminopropyl)-*N* -ethylcarbodiimide hydrochloride (EDC), *N* -hydroxysuccimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma Aldrich (MO, USA). Dulbecco'âs modified Eagle'âs medium (DMEM), fetal bovine serum (FBS), trypsin ethylenediaminetetraacetic acid (EDTA; 0.5% trypsin, 5.3 mM EDTA tetra-sodium), penicillin-streptomycin (100 U/ml) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit were purchased from Invitrogen (Rockville, MD, USA). Biotinylated-siRNA (targeting MDM2), control siRNA, and HiPerfect transfection reagent were purchased from Qiagen (Valencia, CA, USA). Radioimmunoprecipitation assay buffer (RIPA), a lysis buffer, and anti-[beta]Î²-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immun-Star Chemiluminescent Kit Western Blotting Detection System attained from Bio-Rad Laboratories (Hercules, CA, USA). Anti-MDM2 was purchased from Abcam Inc. (Cambridge, MA, USA) and peroxidase-conjugated secondary antibody from Millipore (Billerica, MA, USA). Bicinchoninic acid (BCA) protein assay was purchased from Pierce (Rockford, IL, USA). Streptavidin-coated QDs (SA-QDs) and DAPI (4´,6-diamidino-2-phenylindole) were purchased from Invitrogen Corporation and Chemicon (Temecula, CA, USA) respectively.

*** Synthesis of GC-His**

Partial modification of GC with NAcHis was performed using the modified procedure reported by Park *et al.* [27] . GC (0.5 g) was dissolved in MilliQwater (30 ml) followed by the addition of EDC (2.25 mmol) and NHS (2.25 mmol). At room temperature, NAcHis (0.76 mmol) was added slowly to the reactant solution and was left stirring overnight. The resultant solution was filtered (Amicon, YM-10 membrane) three times and resuspended in MilliQwater (10 ml) each time. The entire dispersed solution was then dialyzed against MilliQwater for 24 h. The final product was freeze-dried and characterized at room temperature by ^{1}H-NMR using a Varian INOVA 500 MHz NMR spectrometer.

*** Preparation of GC-His/siRNA-QDs nanoparticles**

In order to monitor biotinylated siRNAs targeting the MDM2 (GenBank: Accession No. NM_002392), SA-QDs were incubated with biotinylated siRNAs for 1 h at room temperature. SA-QDs were mixed in 1:1 and 1:5 molar ratios with biotinylated siRNAs in RNase free water to achieve a final concentration of 0.1 µM of QDs to 0.1 or 0.5 µM of siRNAs in a final reaction volume. The mixtures were inclubated for 1 h in a tube rotator at room temperature. siRNA-QD conjugates were then purified by two-step ultrafiltration (100 k× Nanosep exclusion filter, Pall Life Sciences, Hampshire, UK) to remove nonconjugated siRNAs. GC-His was dissolved in MilliQwater at a final concentration of 1 mg/ml (stock solution). The siRNA-QDs with a ratio of either 1:1 or 1:5 was added to the GC-His solution under stirred conditions and incubated for 30 min to allow nanoparticle formation (Figure 1). The resultant solution was dialyzed three times in MilliQwater at 4°C and then lyophilized.

*** Gel retardation assay**

The binding of siRNA with QDs was demonstrated by a gel electrophoresis shift in a 0.8% (w/v) agarose gel without ethidium bromide. Samples with different molar ratios, defined as the molar ratio of QDs to siRNA (1:1 and 1:5), were loaded into the gel, and electrophoresis was carried out at 40 V for 120 min running with a tris-borate-EDTA buffer (4.45 mM Tris-base, 1 mM sodium EDTA, 4.45 mM boric acid, pH 8.3). QDs and siRNA-QD bands were visualized using a UV transilluminator at 365 nm.

*** Measurement of nanoparticle size & surface charge**

The hydrodynamic diameter of nanoparticles was measured by dynamic light scattering (DLS) (ΖÎPlus Particle Sizer Ver. 4.11; Brookhaven Instruments Corp). The nanoparticles were suspended in 1 ml distilled water and subjected to zeta-potential measurements on a ΖÎ Potential Analyzer Ver. 3.57 (Brookhaven Instruments Corp). The measurements were carried out in automatic mode and the values were presented as average values of five runs. Transmission electron microscopy (TEM) measurements were performed to image the size and shape of GC-His/siRNA-QD nanoparticles. One drop of a dilute solution of nanoparticles (1 nM)

was spread on top of carbon-coated copper grids and left to dry in air. Samples were then visualized using a JEOL 2010 TEM microscope operating at 200 kV. The nanoparticles were characterized and used without further treatment.

*** Cell culture & transfection**

Human breast cancer, MCF-7 cells (ATCC, Rockville, MD, USA) were cultured in DMEM supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin and 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 5% CO_2 . At 50-80% confluency, MCF-7 cells were trypsinized and diluted to appropriate concentration with fresh medium. Cells were treated with MDM2-siRNA-QDs and GC-His/siRNA-QDs at ratios of 1:1 and 5:1. Transfection of siRNA-QDs or mock siRNAs was performed with HiPerfect transfection reagent (Qiagen) according to the manufacturer'âs instructions.

*** Cytotoxicity assay**

The MTT assay was performed to determine the cytotoxicity of the transfection agents, siRNA-QDs complexes and nanoparticles containing siRNA-QDs. A total of 2×10^4 cells/cm^2 were seeded for 24 h in a 96-well plate and allowed to attach for 24 h. Cells were then treated with MDM2-siRNAs, QDs, siRNA-QDs and GC-His/siRNA-QDs. After 24 h of transfection, cells were assayed by MTT assay kit (Invitrogen). Briefly, treatment solutions were replaced with 100 µl fresh medium to which 10 µl of 12 mM MTT solution was added. After 4 h of incubation at 37°C, 100 µl of SDS-HCl solution was added to each well, followed by further incubation at 37°C. The optical density of samples was determined by a microplate reader (Bio-Rad 3550) at 570 nm.

*** Cellular uptake measurements by immunofluorescence**

MCF-7 human breast cancer cells were cultured in an eight-well plate with a density of 1×10^4 cells in each well and for 24 h. The cells were then transfected with QDs, siRNA-QD complexes and nanoparticles containing siRNA-QDs with different ratios. Following transfection, cells were washed with phosphate buffered saline containing 0.1% BSA and fixed in 4% paraformaldehyde. Finally, cell nuclei were labeled by 2 min incubation with DAPI at a working dilution of 1:1000. The samples were mounted and uptake of siRNA-QDs and nanoparticles were monitored by a Zeiss Confocal microscope (Zeiss LSM 510, Germany).

Imaris 6.3.1 software (Bitplane) was used to measure the total siRNA-QDs content inside MCF-7 cells. A series of optical sections from individual samples recorded from the confocal microscope were loaded into the Imaris program. Individual nuclei (blue) and nanoparticles (red) were defined manually by using the Surpass "add new isosurface" function. The number and intensity of fluorescence of each voxel within the defined nanoparticle was calculated by the software as an average intensity sum. The intensity sums therefore reflect the relative siRNA-QD content.

*** Detection of protein expression by western blotting**

MCF-7 breast cancer cells were seeded 24 h before transfection at a density of 6×10^4 cells per well of a 24-well plate. After transfection, the cells were harvested, and the cell pellets were resuspended in RIPA lysis buffer (Santa Cruz Biotechnology). The cell debris was removed, and the protein concentration was determined with the BCA protein assay. Samples of cell extracts containing 40 µg of protein were analyzed by SDS-PAGE and transferred to nitrocellulose membrane filter. MDM2 protein was detected with anti-MDM2 antibody and peroxidase-conjugated secondary antibody. In addition, [beta]Î²-actin was detected with anti-[beta]Î²-actin and peroxidase-conjugated secondary antibody. The antigen-antibody complexes on the filter were visualized with the Immun-Star Chemiluminescent Kit Western Blotting Detection System. Each sample was tested in triplicate. The absolute intensity of the sample band was calculated by Quantity One software available on the Versadoc instrument. The relative intensity of each band was measured by dividing the absolute intensity of each sample band by the absolute intensity of the standard.

Results & discussion

Glycol chitosan was successfully functionalized with *N* -acetyl-histidine by the formation of an amide bond as shown in Figure 2. ^{1} H-NMR confirmed the synthesis of GC-His through the characteristic peaks of the imidazole ring protons of histidine appearing at approximately 6.0 and 8.0 ppm. In order to visualize the transfection and allocation of siRNA in the cytosol of MCF-7 cells, 21-mer antisense oligonucleotide (siRNA) probes biotinylated at the 5´-end were successfully coupled to streptavidin-coated NIR QDs (SA-QDs). QDs used in this study are decorated with 5-10 streptavidin molecules on their surface, each of which has up to four biotin binding sites.

Streptavidin-QDs were functionalized with biotinylated siRNA targeting MDM2 using either a 1:1 or 5:1 molar ratios (siRNA:SA-QDs) with the intention to illustrate if there will be a considerable variation in cellular uptake and protein inhibition among the two chosen molar ratios. The nature of the streptavidin-biotin self-assembly provides a robust and inert linkage between QDs and siRNAs. The QD-probe conjugation was confirmed by agarose gel electrophoresis and zeta-potential measurements (Figure 3). Unconjugated QDs have no ionic charge and thus only migrated slightly from the well due to the low percentage of gel (Figure 3, lane 4). As for the conjugated QDs, the gel band moved considerably and showed a differential gel shift proportional to the ratio of probe:QD used in the conjugation reaction (Figure 3, lanes 1-3), since greater amounts of probe conjugated per QD will create a greater ionic charge.

Zeta-potential measurements of SA-QDs verified that the overall charge is neutral (Figure 3). To obtain a negatively charged surface and electrostatically self-assemble siRNA-QDs with the positively charged GC-His, the surface charge of the QDs was modulated by varying the molar ratio of siRNAs. Conjugated biotinylated siRNA to SA-QDs (siRNA-QDs) with molar ratios of

1:1 had a zeta-potential value of -11.84 mV, but when increasing the probe:QD ratio to 5:1 a more negative zeta potential (-32.31 mV) was observed, hence these results indicate that more siRNAs are bound to the QDs in the 5:1 formulation. After conjugation of siRNA to QDs, laser confocal images of siRNA-QDs pre- and post-treatment with HiPerfect transfection reagent in MCF-7 cells was performed to confirm that the functionalization of QDs to siRNA does not perturb their transfection efficiency (Figure 4). HiPerfect transfection reagent is commonly used to enhance the delivery of siRNA and many groups employ HiPerfect reagent as a control in studies when presenting cationic polymer gene delivery vehicle-based systems. In addition, HiPerfect enables effective siRNA uptake and efficient release of siRNA inside cells, resulting in high gene knockdown even when using low siRNA concentrations [31-33] . A onefold increase in QDs uptake into cell after addition of HiPerfect reagent (red dots in Figure 4) confirms that siRNAs are conjugated on the surface of SA-QDs.

The inversion in the zeta potential (from -32.31 to +20.59 mV) after the self-assembly of GC-His with siRNA-QDs with molar ratios of siRNA-QDs of 1:1 (nanoplex 1:1) verified that the siRNA-QD complexes were encapsulated by GC-His (Figure 3). A greater inversion of zeta potential (+ 57.57 mV) was observed for the nanoplex 5:1 system (5:1; siRNA:QDs). As a result of the difference in surface charge, the nanoplexes were characterized further using TEM, Figure 3) and DLS (Figure 3). Nanoparticle size characterization is normally challenging due to the polydispersity of samples, but analyzing nanoparticles using a combination of methods, such as TEM and DLS, can offer size information on nanoparticles that might be pertinent to their therapeutic performance. TEM revealed that nanoplex 1:1 formed monodisperse spherical-like nanoparticles approximately 60 nm in size, whereas nanoplex 5:1 generated larger nanoparticles around 120 nm (Figure 3). The variation in size between the two nanoplexes was also consistent with the DLS measurements. The average diameter was found to be 240 nm for nanoplex 1:1 and 450 nm for nanoplex 5:1 (Figure 3). Generally, polymers are not visible to the electron microscope without heavy-metal staining because these composites do not deflect the electron beam adequately. Moreover, the TEM measurements are performed under dry conditions, as oppose to DLS measurements where the polymer is fully hydrated and the size of the nanoparticle is based on the hydrodynamic radii. Therefore, due to the low deflection and hydration of GC-His, a fourfold increase in nanoplex 1:1 and 5:1 diameters has been observed between TEM and DLS measurements. MCF-7 human breast cancer cell viabilities in the presence of siRNA-QDs (either free or entrapped within GC-His) in FBS was also studied, along with other various nanoparticle formulations (Figure 3). A total of 89.5 and 78.2% of cells were viable in the presence of GC-His and GC-His/siRNA-QD nanoparticles, respectively. A considerable increase in cytotoxicity for siRNA-QDs treated with HiPerfect transfection reagent, SA-QDs and bare siRNA-QDs nanoparticles (68.8, 60.3 and 72.7%, respectively) was observed, signifying the protective measures that are acquired by GC-His to camouflage the adverse effects of both QDs and siRNAs.

On the surface of the cell membrane reside negatively charged moieties that favor interaction with positively charged nanoplexes, which then promote their uptake via fluid phase endocytosis. In addition, it has been shown that nanocarriers with hydrodynamic diameters between 200 and 500 nm can be endocytosed into the cell and afterwards can permeate through the nuclear pores into nuclear membranes [34] . Since the nanoplexes size are in this range and positively charged; the nanoplexes prepared with both molar ratios could therefore be endocytosed.

The effect of these parameters on cellular uptake efficiency of GC-His/siRNA-QDs self-assembled nanoparticles was assessed by confocal laser scan microscopy. The GC-His nanoparticles were used as controls instead of commonly used GC nanoparticles since both the targeting and tracking capabilities of nanoplexes could be addressed while the transfection efficiency could be compared to polyplex systems reported in the literature [35,36] . The initial investigation with siRNA-QDs in the absence of GC-His revealed that very low amounts of siRNAs are taken up by MCF-7 human breast cancer cells after 24 h incubation (Figure 4). A drastic increase in the internalization of siRNA by MCF-7 human breast cancer cells was observed with the nanoplex 1:1 system, whereas the use of the larger size 5:1 nanoplex retarded the uptake of siRNA-QDs. Thus, a greater saturation of biotin binding sites was sub-optimal when used to visualize the delivery of siRNA into MCF-7 cells. Even though the variation in molar ratios has influenced the uptake efficiency of our nanoplexes, the 5:1 nanoplexes were still able to be endocytosed into the cells in greater quantity than bare siRNA-QDs. These results clearly show a good balance between surface charge and size is crucial to obtain optimal uptake. Previous work [28] , where chitosan was employed as the delivery vehicle, experienced minimal uptake of siRNA due to the low solubility of chitosan under neutral and alkaline conditions. These results suggest not only that the nanoparticle size and charge contribute to the uptake efficiency, but also the relatively high solubility of GC-His (delivery vehicle) at various pH values plays an important role as it is seen with the 5:1 nanoplex. As a result, the large enhancement in nanoparticle uptake satisfies two of the required properties of GC-His/QD to serve as a multifunctional delivery vehicle.

To test the initial hypothesis, whether GC-His after fluid phase endocytosis can promote the release of the nanoplexes from the endosome vesicles into the cytosol before it is trafficked to enzyme rich lysosomes, we resorted to 3D imaging. Zeta-sectional images were collected in order to reconstruct a 3D image of the MCF-7 breast cancer cells. Cell nuclei were stained with DAPI blue for the localization of siRNA-QDs. Zeta-stack images from cells treated with siRNA-QDs (negative control) and with multifunctional nanoplex vehicles were loaded into the Imaris 6.3.1 imaging software program. In the absence of GC-His, a small amount of siRNA-QDs were found on the inside periphery of the cell membrane, whereas siRNA-QDs encapsulated with GC-His were found to be localized in the cytosol of the cells (Figure 5). By taking the number and intensity of red fluorescence of each voxel within the cell provided by NIR QDs, an average sum of volume intensity was calculated by the software. The average intensity sums therefore correlated to the relative content of siRNA-QDs inside the cells. This semiquantitative analysis

indicated a two order of magnitude difference between the bare siRNA-QDs and the GC-His coated siRNA-QDs (Figures 4 & 5). This provided the evidence that GC-His promoted the proton sponge effect inside the endosomes, which led to the siRNA-QD release into the cytosol (Figure 6A). In addition, as could be seen in Figure 2, despite the fact that nanoplex 5:1 had fewer uptakes than nanoplex 1:1, it was still possible to localize them inside the cytosol of cells and divert their sequestration onto lysosomal compartments.

To demonstrate the ability of the multifunctional nanoplex system to enhance the inhibitory effect of siRNA, MCF-7 human breast cancer cells were treated with mock siRNA (negative control), siRNA targeting the MDM2 receptor loaded with GC-His 5:1, HiPerfect transfection reagent with siRNA (positive control), siRNA loaded with GC-His 1:1, QD-siRNA loaded with GC-His 1:1, and QD-siRNA loaded with GC-His 5:1 for 24 h. The expression of MDM2 protein was then assessed by western blotting analysis. As shown in Figure 6B, siRNA loaded with GC-His strongly inhibited the expression of MDM2 protein, whereas mock siRNA (random siRNA) did not inhibit the expression of MDM2 as was expected. After normalizing the band intensities to [beta]Î²-actin, 73 and 64% reduction in gene expression was observed for siRNA complexed with GC-His in comparison to the negative and positive controls, respectively (Figure 6). As for siRNA functionalized with QDs loaded within GC-His partially inhibited the expression of MDM2 after 24 h but still experienced a higher amount of inhibition as opposed to siRNA loaded along with HiPerfect Transfection reagent. The benefit of the commercially available HiPerfect transfection reagent is that only a moderate amount of siRNA is needed for gene silencing. However, the main disadvantage is its toxicity for *in vivo* applications [37] . It was not surprising to detect a reduced amount of gene knockdown with the developed nanoplex system containing QDs as with ones lacking QDs, this is because of the lower availability of siRNAs loaded within the nanoplex system, which is hampered by the large size of QDs and number of binding sites. In retrospect, these results suggest that the presence of QDs do not inherently hinder the activity of siRNAs but rather slightly effect their loading efficiency inside GC-His. Ultimately, this nanoplex system offers an excellent balance between gene silencing efficacy and toxicity while employing QDs as a self-tracking tool to guide us on the exact allocation of siRNAs inside the cell.

Conclusion

A promising model for a gene delivery vehicle should combine different functional moieties, such as: gene shielding; cellular recognition; cellular uptake; endosomal escape; the ability to image the localization of gene inside the cell; and gene inhibition. Here, the ability of a robust and stable nanoplex system, which encompasses all the above moieties, is demonstrated. In addition to its multifunctionalilty, this nanoplex system transfects siRNA in higher amounts and results in significant knockdown of the expression of the tumor suppressor gene *MDM2* in comparison with siRNAs loaded with HiPerfect transfection reagent. Moreover, GC-His can function as a superior carrier with large surface area and of most important attribute is having high solubility at various pH. The developed gene-delivery system is inexpensive and easy to

functionalize through self-assembly principles with either biological and/or organic molecules. Therefore, it could potentially be used as a powerful tool for magnetic resonance imaging application and biodistribution studies through the inclusion of superparamagnetic iron oxide nanocrystals or other NIR metal nanoparticles allowing for noninvasive methods to monitor the *in vivo* delivery of siRNAs.

Executive summary

GC-His/siRNA-quantum dot nanoplexes

* Glycol chitosan was modified with *N* -acetyl-histidine (GC-His) using EDC/NHS chemistry.

* Streptavidin-coated near-infrared quantum dots (QDs) were functionalized with biotinylated siRNA targeting MDM2 (siRNA-QDs).

* siRNA-QDs were self-assembled with GC-His forming 120 nm size positively charged nanoparticles in solution.

MCF-7 breast cancer cells

* Cytotoxcity assay verified that exposure of nanoplexes to cultured human breast cancer cells had minimal toxicity.

* 3D Immunofluorescence showed that siRNA-QDs encapsulated with GC-His had higher uptake efficiency by MCF-7 cells than bare siRNA-QDs and were localized in the cytoplasm.

* GC-His not only served to protect the siRNA but enhanced their internalization into MCF-7 cells and promoted the proton sponge effect.

Therapeutic efficiency

* Western blot analysis revealed that siRNAs loaded within GC-His showed a marked reduction of MDM2 protein expression 24 h after transfection, as opposed to mock siRNA.

* Furthermore, GC-His offered better MDM2 inhibition than the commercial transfection reagent HiPerfect.

CAPTION(S):

Figure 1. Synthesis of nanoplex *N* -acetyl-histidine on GC-His with a siRNA-quantum dot conjugate.

Streptavidin-coated QDs were mixed in various moral ratios with biotinylated siRNA for 1 h at room temperature. Purified siRNA-QDs conjugates were then added to the GC-His solution for 30 min to form nanoparticles.

GC-His: *N* -acetyl-histidine on glycol chitosan; QD: Quantum dot.

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Author Affiliation(s):

[1] Department of Biomedical Engineering, McGill University, 3775 University Street, Montreal, Quebec, H3A2B4, Canada

[2] Center for Biorecognition & Biosensors, McGill Institute for Advanced Materials, Montreal, Quebec, Canada

[3] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada.
maryam.tabrizian@mcgill.ca

Author Note(s):

[dagger] *Author for correspondence*

** *Authors contributed equally*

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